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(54) Title: REGULATORY CASSETTE FOR EXPRESSION VECTORS

(57) Abstract

Recombinant DNA, e.g. in the form of a plasmid, comprising a cassette as derived from a *Pseudomonas* TOL plasmid, the cassette comprising a combination of the *xylR* gene and an operator promoter region stimulated by the *xylR* gene product. The cassette can be used to regulate gene inserts, to give expression under the control of widely-available aromatic compounds such as toluene.

REGULATORY CASSETTE FOR EXPRESSION VECTORS

Field of the Invention

This invention relates to a regulatory cassette (or unit) which can be used to construct expression vectors which can be transferred into and maintained in a variety of microorganisms. Such expression vectors, under the control of the cassette, enable the microorganisms to produce desirable biological products in an efficient manner.

Background of the Invention

Bacterial expression systems which are controlled by the insertion of regulatory genes are known. For example, there are systems which are designed for the regulation of *E. coli* genes. However, there are few expression vectors for gram-negative, non-enteric bacteria. An example of such an organism is *Xanthomonas* which produces the extracellular polysaccharide xanthan used in microbiologically-enhanced tertiary oil recovery. Xanthan gum also has widespread use as a food additive and as a lubricating agent. Other examples of organisms which secrete products of commercial importance are *Acinetobacter* which produces polysaccharides which can be used for cleaning oil spills, *Azctobacter* which synthesises compounds of use as food additives, *Erwinia* which secretes pectins of use for clarifying fruit juices, and *Pseudomonas* which has a wide range of metabolic activities which can be employed in bio-transformation processes.

There are few expression vectors which are regulated by cheap, readily-available compounds such as toluene or benzyl alcohol. Such compounds act via regulatory genes such as *xylR* (present in TOL plasmids). The nucleotide sequence of the *xylR* promoter region in the archetypal

downstream of p_u, in the presence of simple and cheap aromatic compounds, e.g. having 6-12 carbon atoms, such as toluene or benzyl alcohol.

By virtue of the present invention, genes coding for certain functions of gram-negative bacteria can be expressed in a controlled manner in the same host as that from which they were originally cloned. As a result, the cloned genes are well expressed and are unlikely to produce products which are detrimental to the host bacteria's physiology. Alternatively, the cassette can also be used for heterologous gene expression in gram-negative bacteria.

Description of the Invention

The expression vectors of the present invention may be based on known plasmids such as RSF 1010, into which a regulatory cassette based on xylR/p_u is inserted. All DNA sequences necessary for the construction of plasmids (to carry the regulatory cassette) which exemplify the invention have been physically mapped and analysed.

An illustrative expression vector of the present invention comprises a cassette which has been isolated from the TOL plasmid pWW53. The regulatory cassette, which is generally required to be substantially free of functional DNA other than the xylR gene and p_u, can be isolated in a number of ways, using techniques known to those skilled in the art.

A process for preparing the cassette comprises cleaving a Pseudomonas TOL plasmid (with restriction endonucleases) to obtain a fragment containing most or all of the combination. If necessary or desired, a further step comprises removing any genes and/or undesirable restriction sites from the fragment other than the xylR gene by further gene manipulations. For example, deletion may comprise one or more DNA sequences

inserted as EcoRI fragment into the EcoRI site of pPLGN1 which already carries λp_L and the cI857 gene. KpnI and SacI restriction fragments can be inserted downstream of p_u. In this vector system, the initial induction is achieved by raising the temperature, say from 28°C to 42°C, leading to the over-expression of XylR from λp_L. This over-expression of XylR in turn leads, in the presence of inducers (e.g. toluene or benzyl alcohol), to a high level of transcription of a gene inserted downstream of p_u. This cascade induction system is particularly useful because, if the native weak xylR promoter is retained, the two-level expression vector can be used to give moderate induction in the presence of the hydrocarbon inducers during exponential growth and, at the end of the growth phase, very high levels by increasing the temperature to 42°C.

The efficiency of both vectors can be monitored by inserting appropriate indicator genes into the insertion sites such as the CDO (catechol 2,3-dioxygenase) or lacZ gene.

The usefulness of vector systems of this second type can be further demonstrated by inserting the genes necessary for the conversion of toluene to benzoate downstream of p_u. The efficient conversion of aromatic hydrocarbons into carboxylic acids is a potentially valuable bio-transformation process.

The third type of vector is a highly repressible expression vector. It is constructed in a similar manner to the second type of vector, but with the transcriptional orientation of the xylR/p_u cassette facing towards λp_L at the pPLGN1 EcoRI site; again SacI and KpnI restriction fragments can be inserted. The transcription of a gene cloned into these sites in either orientation can be highly repressed by inducing the promoter downstream of the insert, thus generating high

the cloning vector pUC19 in order to obtain the first plasmid shown in the drawing (described as pEHK11). This plasmid carried several superfluous genes from the TOL pathway in the upstream region and a short interfering DNA segment downstream, all of which should be removed. The TOL genes xyl LEGF were deleted by SacI subcloning of pEHK11, to obtain pEHK13.

pEHK13 was partially restricted with BamH1 and Bg1II and a 5.2 kb region was obtained which carried no functional TOL genes upstream of xy1R. This DNA construct (pEHK15) carried a unique BamH1 site downstream of the coding region of xy1R and, less than 50bp to its right, a BstEII site (one of three on this insert).

The purpose of succeeding steps was to obtain both xy1R and p_u on a BstEII fragment, because there would thus be very convenient border sequences on either side: on the left-hand end, only the promoter and ribosome binding site (PBS) of xy1R; on the right-hand end, p_u and its RBS but almost no non-essential and hence potentially deleterious DNA. To this end, pEHK15 DNA, which has three BstEII sites, was taken, restricted at the unique BamH1 site which was known to be located downstream of the xy1R gene and only about 50 nucleotides away from the central BstEII site (H. Keil et al., J. Bacteriol. 169, supra) and subsequently treated with exonuclease Ba131 at a rate which allowed the continuous removal of between 0.1 to 1.0 kb in both directions. The DNA was then blunt-ended by PolIk treatment and religated with T4-DNA ligase. After transformation into E. coli ED8654, 48 colonies were analysed for their DNA profile and found to have deletions ranging in length from 0.1 to 1.0 kb. They had all lost their central BstEII site and hence gave rise to a unique BstEII fragment of variable length of between 3.9 and 3.0 kb containing all or part of xy1R + p_u (pEHK250 series). DNA from 6 representative clones

CLAIMS

1. A regulatory cassette as derived from a Pseudomonas TOL plasmid, which comprises the xylR gene in combination with a promoter stimulated by the xylR gene product.

2. A regulatory cassette according to claim 1, which comprises no genes from the TOL plasmid other than the xylR gene.

3. A regulatory cassette according to claim 1 or claim 2, in which the gene-promoter combination is as found in Pseudomonas putida.

4. A regulatory cassette according to claim 3, in which the gene-promoter combination is as found in plasmid pWW53.

5. DNA, e.g. in the form of a plasmid, comprising a heterologous cassette according to any preceding claim.

6. An expression vector comprising a replicon and a regulatory cassette according to any of claims 1 to 4, the cassette allowing improved expression of a gene insert in the vector.

7. A vector according to claim 6, which contains a promoter additional to and heterologous with the promoter stimulated the xylR gene product.

8. A vector according to claim 7, in which the promoter is from bacteriophage λ.

9. A vector according to any of claims 6 to 8, which additionally comprises a gene for a thermolabile repressor protein.

10. A vector according to any of claims 6 to 9, which additionally comprises a gene transcribing anti-sense mRNA which, if induced, represses the xylR gene and, if repressed, allows expression of the xylR gene.

11. A vector according to any of claims 6 to 10, which has a multi-cloning site downstream from the gene-promoter combination.

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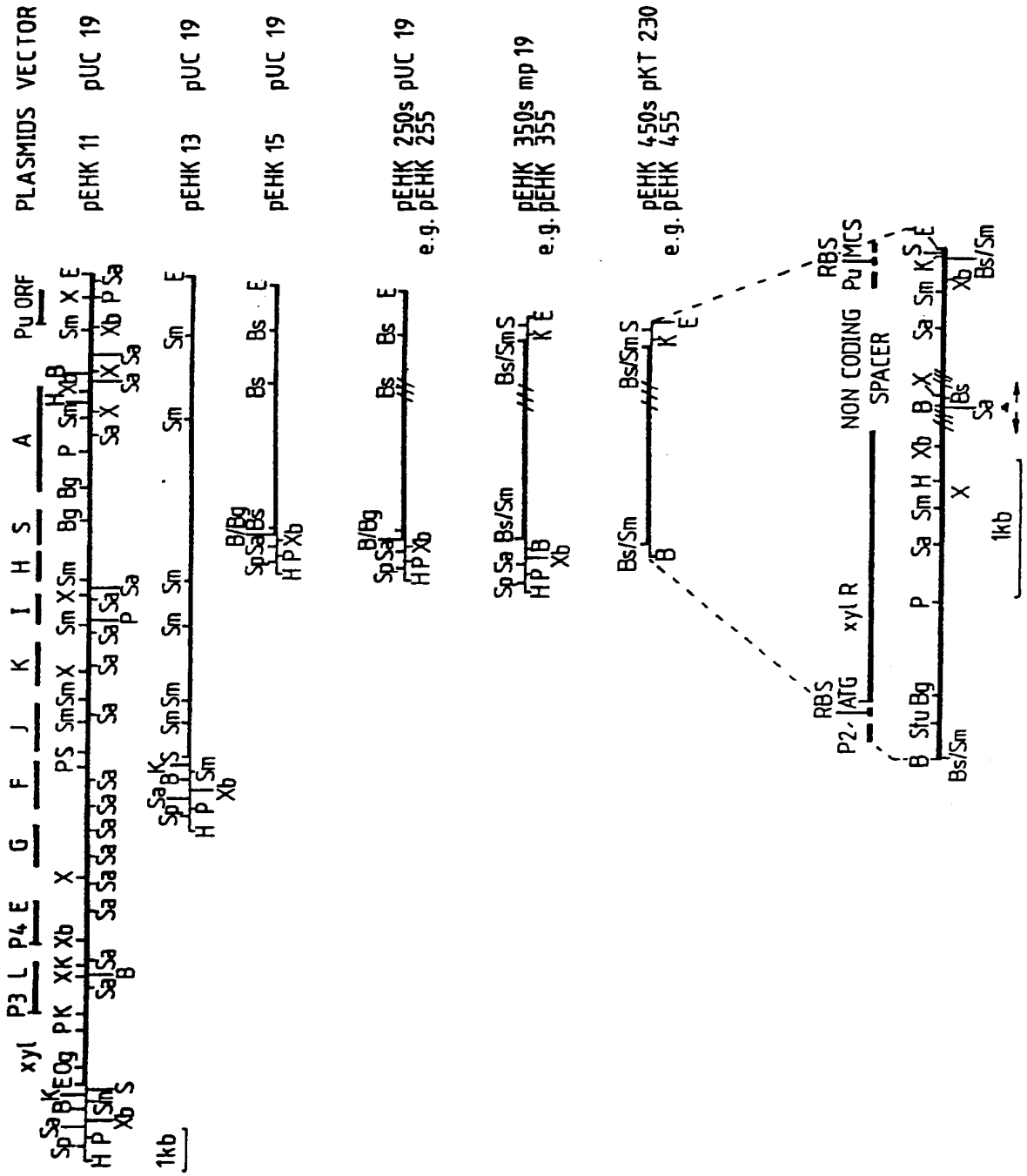

SUBSTITUTE SHEET

International Application No. PCT/GB 89/00341

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Febs Letters, vol. 226, no. 2, January 1988, Elsevier Science Publishers B.V., (Biomedical Division), (Amsterdam, NL), J.L. Ramos et al.: "Broad-host range expression vectors containing manipulated meta-cleavage pathway regulatory elements of the TOL plasmid", pages 241-246 see the whole article --	1-17
A	Chemical Abstracts, vol. 105, no. 17, 27 October 1986, (Columbus, Ohio, US), N. Mermod et al.: "Vector for regulated expression of cloned genes in a wide range of Gram-negative bacteria", see page 197, abstract 147488k, & J. Bacteriol. 1986, 167(2), 447-54 --	1-17
A	Journal of Bacteriology, vol. 164, no. 2, November 1985, American Society for Microbiology, (Baltimore, US), H. Keil et al.: "Evolutionary conservation of genes coding for meta pathway enzymes within TOL plasmids pWWO and pWW53", pages 887-895 see the whole article cited in the application --	1
A	Proc. Natl. Acad. Sci. USA, vol. 84, no. 15, August 1985, (Washington, DC, US), S. Inouye et al.: "Expression of the regulatory gene xyIS on the TOL plasmid is positively controlled by the xyIR gene product", pages 5182-5186 see the whole article --	1,3,4-6
A	EP, A, 0242220 (NOVO INDUSTI A/S) 21 October 1987 see the whole document ----------	1-17

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